blood

2012 120: 2118-2126
Prepublished online July 18, 2012;
doi:10.1182/blood-2012-02-411827

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

High expression of lymphoid enhancer-binding factor-1 (*LEF1*) is a novel favorable prognostic factor in cytogenetically normal acute myeloid leukemia

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Lymphoid enhancer-binding factor-1 (*LEF1*) is a key transcription factor of Wnt signaling. We recently showed that aberrant *LEF1* expression induces acute myeloid leukemia (AML) in mice, and found high *LEF1* expression in a subset of cytogenetically normal AML (CN-AML) patients. Whether *LEF1* expression associates with clinical and molecular patient characteristics and treatment outcomes remained unknown. We therefore studied *LEF1* expression in 210 adults with CN-AML treated on German AML Cooperative Group trials using microarrays. High *LEF1* expression (*LEF1*^{high}) associated with significantly better relapse-free survival (RFS; $P < .001$), overall survival (OS; $P < .001$), and event-free survival (EFS; $P < .001$). In multivariable analyses adjusting for established prognosticators, *LEF1*^{high} status remained associated with prolonged RFS ($P = .007$), OS ($P = .01$), and EFS ($P = .003$). In an independent validation cohort of 196 CN-AML patients provided by the German-Austrian AML Study Group, *LEF1*^{high} patients had significantly longer OS ($P = .02$) and EFS ($P = .04$). We validated the prognostic relevance of *LEF1* expression by quantitative PCR, thereby providing a clinically applicable platform to incorporate this marker into future risk-stratification systems for CN-AML. Gene-expression profiling and immunophenotyping revealed upregulation of lymphopoiesis-related genes and lymphoid cell-surface antigens in *LEF1*^{high} patients. In summary, we provide evidence that high *LEF1* expression is a novel favorable prognostic marker in CN-AML. (*Blood*. 2012;120(10):2118-2126)

Introduction

The Wnt signaling pathway is a critical regulator of stem cell function in healthy tissues and cancer, including acute myeloid leukemia (AML).[1] Studies in humans and mice have demonstrated that the Wnt pathway is essential for maintenance, activation, and proliferation of normal hematopoietic stem cells,[1] and that aberrant Wnt signaling can lead to expansion of leukemic stem cells in myeloid and lymphoid neoplasias.[2-5] Activation of the transcriptional coactivators β-catenin and γ-catenin, the main downstream effectors of canonical Wnt signaling, is linked to enhanced leukemogenic potential and self-renewal of putative leukemic stem cells.[5-7]

Lymphoid enhancer-binding factor-1 (*LEF1*) is a member of the LEF1/T-cell factor (TCF) family of transcription factors. During canonical Wnt signaling, LEF1/TCF proteins directly interact with β-catenin to induce expression of target genes, including the cell-cycle regulators cyclin D1 and c-myc.[1] In addition, *LEF1* has roles in normal hematopoiesis and leukemogenesis that are independent of its involvement in Wnt signaling.[8,9] Our group previously demonstrated that ordered expression of *Lef-1* is necessary for normal hematopoietic stem cell function in mice, and that *Lef-1* overexpression induces AML which is propagated by leukemic stem cells with lymphoid characteristics.[10] A subset of patients with cytogenetically normal AML (CN-AML) express high levels of *LEF1*,[10] but associations between *LEF1* expression and other clinical and molecular patient characteristics and outcomes are unknown so far. Here, we demonstrate for the first time that high *LEF1* expression is a favorable prognostic factor in patients with CN-AML in 2 independent, relatively large AML patient cohorts when measured by microarray techniques or quantitative PCR (qPCR), and is associated with distinct molecular and immunophenotypic characteristics.

Methods

Patients

We studied 210 patients with previously untreated CN-AML (median age, 59 years; range, 17-83 years) who were treated on 2 consecutive phase 3 trials of the German AML Cooperative Group (AMLCG-1992 and AMLCG-1999 [clinicaltrials.gov identifier NCT00266136]) between 1999 and 2004.[11,12] One hundred nine patients (52%) were aged < 60 years (younger patients) and 101 patients (48%) were ≥ 60 years (older patients). All patients received

Submitted February 20, 2012; accepted July 12, 2012. Prepublished online as *Blood* First Edition paper, July 18, 2012; DOI 10.1182/blood-2012-02-411827.

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Presented in part at the 51st annual meeting of the American Society of Hematology, New Orleans, LA, December 7, 2009.

The online version of this article contains a data supplement.

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Table 1. Pretreatment characteristics in the primary cohort of 210 CN-AML patients according to *LEF1* expression levels

Variable	*LEF1*^{high}, n = 105	*LEF1*^{low}, n = 105	*P*
Median age, y (range)	57 (17-77)	59 (18-83)	.56
Female sex, no. (%)	59 (56)	63 (60)	.67
Secondary or treatment-related AML, no. (%)	4 (4)	6 (6)	.75
FAB subtype, no.			.21
M0	5	1	
M1	22	35	
M2	32	33	
M4	27	19	
M5	12	13	
M6	6	3	
RAEB-2	1	1	
Median WBC, 10^9/L (range)	21.0 (0.9-216)	44.6 (0.85-486)	< .001
Median BM blasts, % (range)	80 (11-100)	87.5 (17-100)	.02
Median platelet count, 10^9/L (range)	64 (6-280)	48 (9-471)	.04
FLT3-ITD present, no. (%)	25 (24)	61 (58)	< .001
NPM1 mutated, no. (%)	52 (50)	61 (58)	.27
***CEBPA* mutated, no. (%)**	11 (11)	12 (12)	> .99
Monoallelic *CEBPA* mutation	3	8	
Biallelic *CEBPA* mutation	8	4	
Missing data	6	3	
ELN genetic group,[25] no. (%)			.01
Favorable	49 (48)	31 (30)	
Intermediate-I	53 (52)	71 (70)	
Missing data	3	3	
***IDH1* mutated, no. (%)**	8 (12)	10 (12)	> .99
Missing data	40	21	
***IDH2* mutated, no. (%)**	11 (17)	15 (18)	> .99
Codon R140 mutation	11	15	
Codon R172 mutation	0	0	
Missing data	40	21	
***FLT3*-TKD (D835) mutated, no. (%)**	9 (9)	8 (8)	.81
Missing data	1	0	
***MLL*-PTD present, no. (%)**	15 (15)	11 (11)	.41
Missing data	4	2	
High *ERG* expression,* no. (%)	19 (18)	34 (32)	.03
High *BAALC* expression,* no. (%)	52 (50)	53 (50)	1.0
High *MN1* expression,* no. (%)	53 (50)	52 (50)	1.0

CN-AML indicates cytogenetically normal acute myeloid leukemia; FAB, French-American-British classification; WBC, white blood cell count; ITD, internal tandem duplication; ELN, European Leukemia Net; TKD, tyrosine kinase domain; and PTD, partial tandem duplication.

*High *BAALC* and *MN1* expression were defined as an expression level above the median of all samples, respectively, and high *ERG* expression was defined as an expression level above the 75th percentile.[19,26]

cytarabine-based intensive induction and consolidation chemotherapy.[11-13] The diagnosis of a normal karyotype was based on conventional cytogenetic examination of at least 20 metaphases from bone marrow (BM). Patients were characterized for *NPM1*, *CEBPA, IDH1*, and *IDH2* mutations, *FLT3*-internal tandem duplications (*FLT3*-ITD), tyrosine kinase domain mutations (*FLT3*-TKD [D835]), and *MLL* partial tandem duplications (*MLL*-PTD), as described previously.[14-16] An independent validation cohort of 196 younger (< 60 years) CN-AML patients was provided by the German-Austrian AML Study Group (AMLSG; trials AMLSG-HD98A [NCT00146120][17] and AMLSG 07-04 [NCT00151242]). This patient group was characterized for the gene mutations previously mentioned, and for *TET2, ASXL1, DNMT3A, RUNX1*, and *WT1* mutations. All study protocols were in accordance with the Declaration of Helsinki and approved by the institutional review boards of the participating centers, and all patients provided written informed consent.

Microarray analyses

For the primary (AMLCG) cohort, pretreatment BM samples were studied using Affymetrix HG-U133A (n = 154) or HG-U133plus2.0 (n = 56) oligonucleotide microarrays as described previously (for details on microarray data processing, see supplemental Methods, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).[18,19] Patients with *LEF1* expression values above the median of all patients were classified as having high *LEF1* expression (*LEF1*^{high}), and all other patients were considered to have low *LEF1* expression (*LEF1*^{low}). The choice of the median as the threshold for separating patients into 2 groups was based on analyses of outcomes according to quartiles of *LEF1* expression (data not shown). *ERG*, *BAALC*, *MN1*, and *EVI1* expression levels were also determined from the microarray data.[19] In the independent validation cohort provided by the AMLSG, pretreatment BM (n = 142) or peripheral blood (n = 54) samples were studied by independent investigators using cDNA microarrays (n = 130) or Affymetrix HG-U133plus2.0 oligonucleotide microarrays (n = 66), as previously described.[20,21] *LEF1* expression levels were dichotomized at the median of all samples. Analysis of differentially expressed genes and gene set enrichment analysis (GSEA) are described in supplemental Methods. Microarray data are available at the Gene Expression Omnibus database (GEO; accession no. GSE12417, GSE8043, and GSE15434).[22]

Measurement of LEF1 expression by qPCR

LEF1 expression was measured by real-time qPCR in a subgroup of 122 CN-AML patients from the primary cohort for which sufficient material was available. cDNA had been prepared at the time of initial diagnosis using random hexamer primers, and cryopreserved at −80°C. *LEF1* expression was measured using a TaqMan probe-based qPCR assay

recognizing all 4 major human *LEF1* isoforms (Hs01547250_m1; Applied Biosystems), and normalized to *ABL* expression (Hs01104724_mH).[23] To allow comparison of our expression data and thresholds with future studies, expression values were standardized to a calibrator sample (cDNA from the K562 cell line), using the comparative threshold cycle (C_T) method.[24] An analysis of Martingale residuals from univariable Cox models identified a $\Delta\Delta C_T$ value of -2.50 as a clinically informative threshold to separate *LEF1*^{high} and *LEF1*^{low} patients.

Flow cytometry

Multiparameter flow cytometric analysis was performed on BM mononuclear cells from 124 AMLCG patients, using a FACSCalibur flow cytometer (BD Biosciences). Patients were characterized for 28 antigens using 17 triple monoclonal antibody combinations, including CD34/CD2/CD33, CD7/CD33/CD34, CD4/CD13/CD14, and cyTdT/cyCD79a/cyCD3 (conjugated with the fluorochromes FITC, PE, and PE–cyanin-5.1, respectively; Immunotech), and 20 000 events were acquired per specimen.

Statistical analyses

Definitions of clinical endpoints (complete remission [CR], relapse-free survival [RFS], overall survival [OS], and event-free survival [EFS]) are listed in supplemental Methods. The association between *LEF1* expression as a continuous numerical variable and patient outcomes was studied using univariable Cox proportional hazards and logistic regression models. Baseline clinical and molecular characteristics were compared between *LEF1*^{high} and *LEF1*^{low} patients using the Fisher exact test for categorical variables and the Wilcoxon rank-sum test for continuous variables. Time-to-event variables were analyzed according to the Kaplan-Meier method, and *P* values were calculated by the log-rank test. A multivariable logistic regression model was constructed for factors associated with achievement of CR, and multivariable Cox proportional hazards models were used to study factors associated with survival endpoints. No variable selection technique was used, and all variables remained in the multivariable model. All statistical data analyses were performed using the R 2.14.1 software package (R Foundation for Statistical Computing).

Results

Association of *LEF1* expression levels with pretreatment patient characteristics

In the primary (AMLCG) cohort, patients with high *LEF1* expression had lower pretreatment white blood cell counts (WBC; $P < .001$), lower BM blasts percentages ($P = .02$), and higher platelet counts ($P = .04$) than *LEF1*^{low} patients (Table 1). *LEF1*^{high} patients were less likely to carry a *FLT3*-ITD ($P < .001$) than *LEF1*^{low} patients. We found no association between *LEF1* expression and other gene mutations, but *LEF1*^{high} patients were less likely to have high expression of *ERG* ($P = .03$). *LEF1* expression levels did not differ significantly between younger and older patients ($P = .20$).

High *LEF1* expression levels associate with favorable treatment outcomes

The median OS of the entire AMLCG patient cohort was 12.7 months, and the median follow-up according to Korn was 47 months.[27] When *LEF1* expression was analyzed as a continuous variable (supplemental Table 1), higher levels were significantly associated with higher CR rate ($P = .03$), longer RFS ($P = .005$), longer OS ($P < .001$), and longer EFS ($P < .001$). When patients were dichotomized according to their *LEF1* expression levels, *LEF1*^{high} patients showed a trend toward a higher CR rate (70% vs 57%, $P = .09$), and had significantly longer RFS ($P < .001$; Figure 1A), OS ($P < .001$; Figure 1B), and EFS ($P < .001$; Figure 1C) than *LEF1*^{low} patients (Table 2).

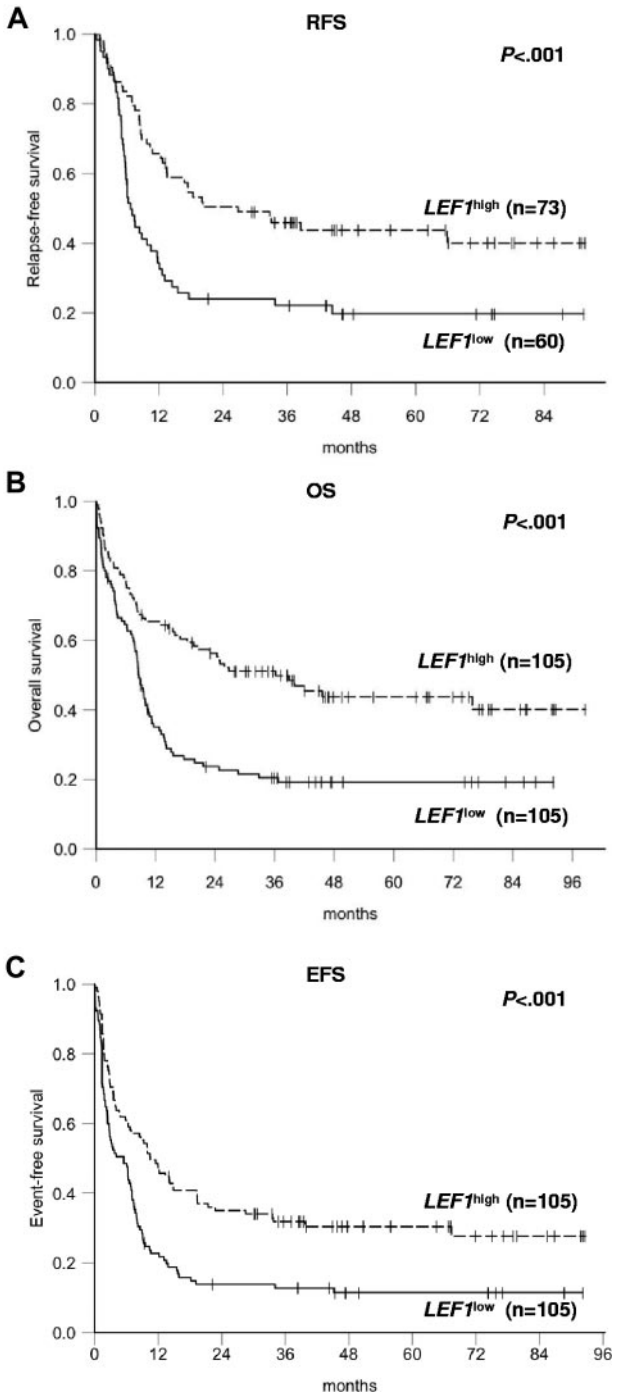

Figure 1. Survival of CN-AML patients according to *LEF1* expression levels. Kaplan-Meier plots of (A) RFS, (B) OS, and (C) EFS in the primary cohort.

Age is one of the most important risk factors in CN-AML. The impact of molecular markers may vary with age,[28] and in our primary cohort, younger (< 60 years) and older patients (≥ 60 years) received different chemotherapy dosages during induction therapy. Therefore, we studied the association of *LEF1* expression with outcomes in both age groups separately. Among younger patients, *LEF1* expression was not associated with attainment of CR ($P > .99$; Table 2). However, younger *LEF1*^{high}

Table 2. Treatment response and survival according to *LEF1* expression in the primary cohort

	All patients, n = 210			Patients younger than 60 y, n = 109			Patients 60 y and older, n = 101		
	LEF1^{high}	*LEF1*^{low}	*P*	*LEF1*^{high}	*LEF1*^{low}	*P*	*LEF1*^{high}	*LEF1*^{low}	*P*
CR rate, %	70	57	.09	70	68	> .99	69	46	.03
RFS			< .001			.01			.004
Median RFS, mo	26.8	6.7		nr	9.8		13.5	5.7	
Estimated RFS at 3 y, % (95% CI)	46 (36-59)	22 (14-36)		61 (48-79)	32 (20-52)		29 (17-49)	8 (2-31)	
OS			< .001			.006			< .001
Median OS, mo	36.2	8.6		75.8	10.7		22.8	7.8	
Estimated OS at 3 y, % (95% CI)	51 (42-62)	20 (14-30)		60 (48-74)	27 (17-42)		42 (30-58)	14 (7-28)	
EFS			< .001			.04			< .001
Median EFS, mo	10.5	5.5		13.7	7.7		9.9	2.4	
Estimated EFS at 3 y, % (95% CI)	32 (24-42)	13 (8-21)		43 (31-58)	22 (13-37)		20 (11-35)	4 (0-13)	

CR indicates complete remission; RFS, relapse-free survival; OS, overall survival; CI, confidence interval; EFS, event-free survival; and nr, not reached.

patients, compared with younger *LEF1*^{low} patients, had significantly longer RFS ($P = .01$; Figure 2A), longer OS ($P = .006$; Figure 2B), and longer EFS ($P = .04$; Table 2). Among older patients, those with high *LEF1* expression had a higher CR rate (69% vs 46%; $P = .03$), longer RFS ($P = .004$; Figure 2C), longer OS ($P < .001$; Figure 2D), and longer EFS ($P < .001$) than *LEF1*^{low} patients (Table 2).

Varying proportions of nonmalignant cells in the BM specimens might confound *LEF1* expression measurements and thereby influence the outcome analyses. Exploratory analyses restricted to patients with a pre-Ficoll BM blast count of > 80% (n = 136) revealed that high *LEF1* expression was still associated with higher CR rate ($P = .008$), longer RFS ($P < .001$), and longer OS ($P < .001$; data not shown).

Prognostic impact of *LEF1* expression in molecular subsets of CN-AML

According to the European Leukemia Net (ELN) reporting system for genetic changes in AML, CN-AML patients are categorized into the ELN Favorable or ELN Intermediate-I genetic category, depending on their *CEBPA*, *NPM1*, and *FLT3*-ITD mutation

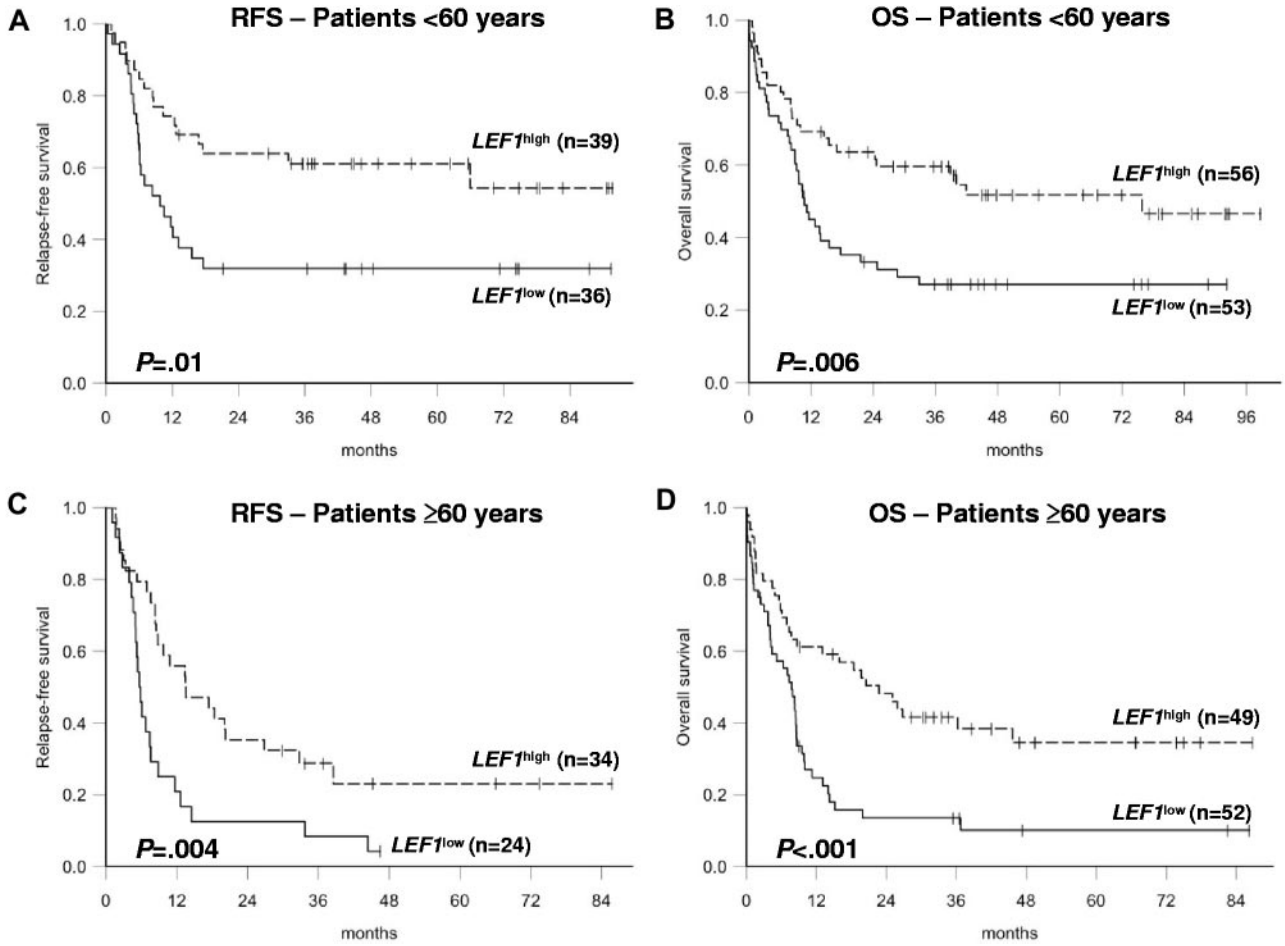

Figure 2. Survival of CN-AML patients according to age group and *LEF1* expression. (A) RFS and (B) OS of CN-AML patients younger than 60 years of age. (C) RFS and (D) OS of CN-AML patients aged 60 years or older.

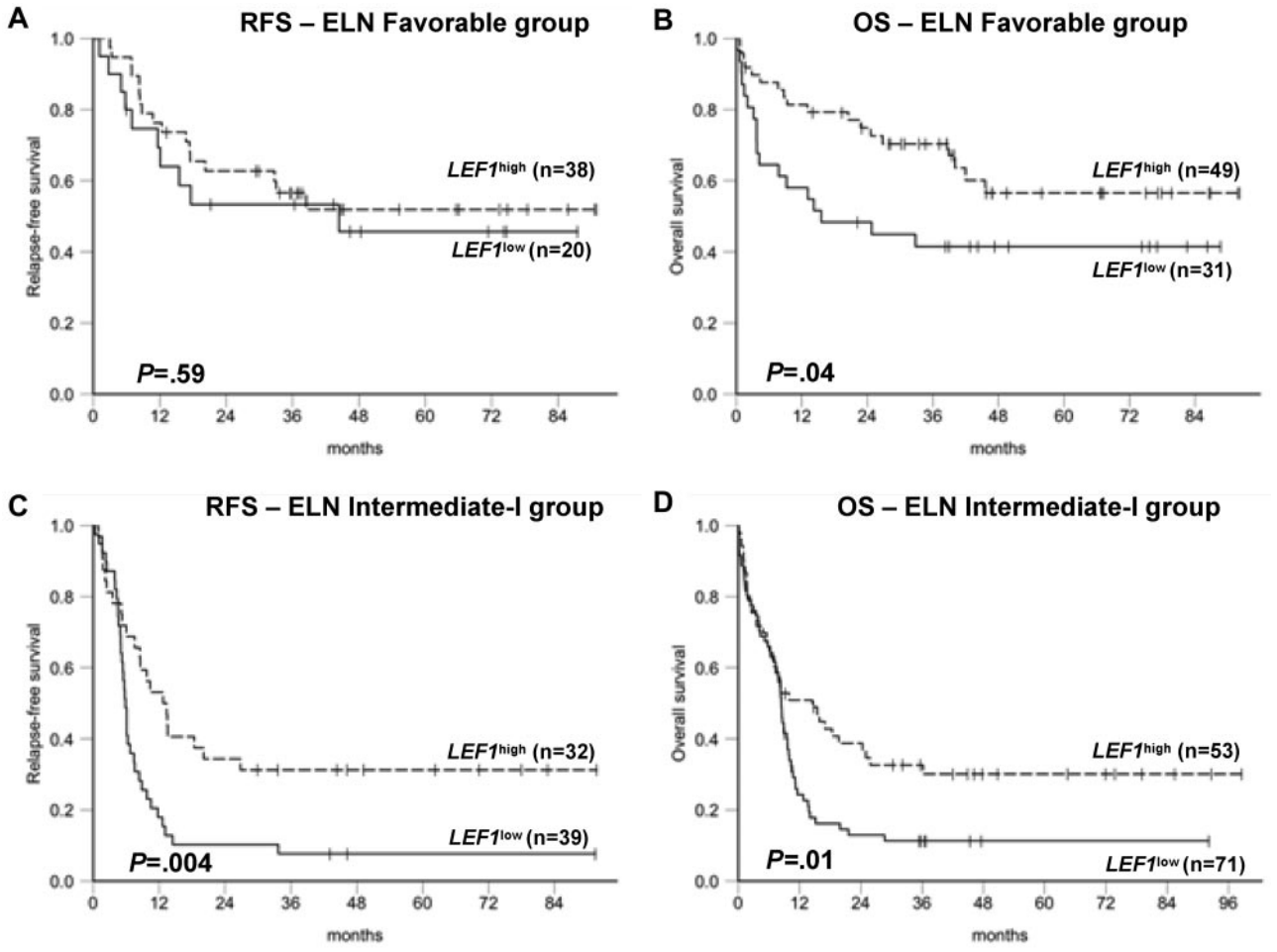

Figure 3. Survival of CN-AML patients according to ELN genetic group and *LEF1* expression. (A) RFS and (B) OS of CN-AML patients in the ELN favorable genetic group. (C) RFS and (D) OS of CN-AML patients in the ELN intermediate-I genetic group.

status.[25] *LEF1* expression differed between the ELN genetic groups. Forty-eight percent of *LEF1*^{high}, but only 30% of *LEF1*^{low} patients belonged to the ELN favorable genetic category ($P = .01$; Table 1). Therefore, we evaluated the impact of *LEF1* expression within the 2 ELN genetic groups separately (supplemental Table 2). In the ELN favorable genetic group, *LEF1* expression was not associated with CR rate ($P = .30$), RFS ($P = .59$; Figure 3A), or EFS ($P = .15$). However, OS of ELN favorable/*LEF1*^{high} patients was significantly longer than for ELN favorable/*LEF1*^{low} patients ($P = .04$; Figure 3B). Within the ELN intermediate-I genetic group, *LEF1* expression was not associated with CR rate ($P = .59$). ELN intermediate-I patients with high *LEF1* had longer RFS ($P = .004$; Figure 3C), longer OS ($P = .01$; Figure 3D), and longer EFS ($P = .01$) than those with low *LEF1*.

LEF1 expression associates with favorable RFS, OS, and EFS in multivariable analyses

We performed multivariable analyses to determine the prognostic significance of *LEF1* expression after adjusting for the impact of other known risk factors. In a multivariable model for CR achievement, *LEF1*^{high} patients showed a trend toward higher odds for achievement of remission ($P = .08$; odds ratio, 1.82; 95% confidence interval, 0.94-3.55). The only factors significantly associated with a higher chance of reaching CR were younger age ($P = .04$) and presence of an *NPM1* mutation ($P = .04$). In a model for RFS, *LEF1*^{high} patients had a 50% lower risk of relapse or death compared with *LEF1*^{low} patients ($P = .007$; Table 3). Other factors significantly associated with longer RFS were younger age and the *NPM1*-mutated/*FLT3*-ITD–negative genotype. In a multivariable model for OS, high *LEF1* expression was associated with a 40% reduction of the risk of death ($P = .01$; Table 3). Other factors associated with longer survival were younger age, the *NPM1*-mutated/*FLT3*-ITD–negative genotype, and biallelic *CEBPA* mutations. Finally, high *LEF1* expression was significantly associated with longer EFS (42% risk reduction; $P = .003$) together with younger age, the *NPM1*-mutated/*FLT3*-ITD–negative genotype, and wild-type *IDH1* (Table 3).

Validation in an independent cohort of CN-AML samples

To validate our findings, we studied an independent cohort of 196 previously untreated younger CN-AML patients provided by the AMLSG. Follow-up for survival was 61.5 months. In the validation cohort, *LEF1*^{high} patients had a significantly lower WBC, lower incidence of concomitant *FLT3*-ITD, and trended toward lower BM blast percentage and younger age at diagnosis, compared with *LEF1*^{low} patients (supplemental Table 3). The CR rate was 80% for *LEF1*^{high} patients and 69% for *LEF1*^{low} patients ($P = .14$; supplemental Table 4). *LEF1*^{high} patients showed a nonsignificant difference in RFS ($P = .18$; supplemental Figure 1A) and had a

Table 3. Multivariable analyses in the primary cohort of 210 CN-AML patients

	RFS, n = 133		OS, n = 210		EFS, n = 210	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
LEF1 expression, high vs low	0.50 (0.30-0.83)	.007	0.60 (0.40-0.90)	.01	0.58 (0.40-0.83)	.003
Age, per 10-y increase	1.28 (1.08-1.53)	.006	1.26 (1.09-1.46)	.002	1.31 (1.14-1.49)	< .001
Sex, male vs female	1.13 (0.69-1.86)	.62	1.08 (0.73-1.59)	.70	1.20 (0.84-1.71)	.31
WBC, logarithmic, per 10-fold increase	1.39 (0.91-2.13)	.13	1.33 (0.96-1.87)	.09	1.24 (0.92-1.70)	.15
Secondary or therapy-related AML, vs de novo AML	nd*		1.43 (0.68-3.02)	.34	0.94 (0.45-1.95)	.87
NPM1 mutated/*FLT3*-ITD negative, vs other genotypes	0.27 (0.14-0.51)	< .001	0.43 (0.25-0.73)	.002	0.38 (0.24-0.62)	< .001
***CEBPA* mutation**						
Monoallelic vs absent	0.38 (0.14-1.05)	.06	0.52 (0.24-1.13)	.10	0.53 (0.26-1.07)	.08
Biallelic vs absent	0.52 (0.16-1.69)	.27	0.38 (0.14-1.00)	.05	0.79 (0.37-1.72)	.55
IDH1 mutation, present vs absent	1.91 (0.81-4.49)	.14	1.80 (0.90-3.61)	.10	1.87 (1.01-3.44)	.04
IDH2 mutation, present vs absent	0.98 (0.49-2.00)	.97	0.78 (0.43-1.41)	.41	0.90 (0.54-1.51)	.69
MLL-PTD, present vs absent	1.15 (0.51-2.62)	.73	1.20 (0.66-2.16)	.56	1.38 (0.80-2.37)	.24
ERG expression, high vs low	1.57 (0.86-2.86)	.14	1.42 (0.91-2.22)	.13	1.42 (0.94-2.14)	.10
BAALC expression, high vs low	0.93 (0.53-1.62)	.79	1.28 (0.80-2.03)	.29	1.07 (0.71-1.63)	.73
MN1 expression, high vs low	1.59 (0.92-2.77)	.10	0.95 (0.61-1.47)	.81	1.28 (0.86-1.92)	.23
EVI1 overexpression, present vs absent	nd*		1.15 (0.55-2.41)	.71	1.23 (0.63-2.40)	.54

Multiple imputations using a predictive mean matching algorithm were used in the case of missing covariables.

CN-AML indicates cytogenetically normal acute myeloid leukemia; RFS, relapse-free survival; OS, overall survival; EFS, event-free survival; HR, hazard ratio; CI, confidence interval; WBC, white blood cell count; ITD, internal tandem duplication; nd, not done; and PTD, partial tandem duplication.

*This variable could not be included in the model for RFS due to the small patient number.

significantly longer OS ($P = .02$; supplemental Figure 1B) and EFS ($P = .04$) than *LEF1*^{low} patients in the validation cohort.

LEF1 expression measured by qPCR discriminates prognostic subgroups in CN-AML

We confirmed the prognostic relevance of *LEF1* expression when measured by qPCR in a subgroup of 122 CN-AML patients from the primary cohort with available material (supplemental Table 5). Microarray and qPCR expression measurements showed a highly significant correlation ($r = 0.69$; $P < 1 \times 10^{-15}$).[29] Higher *LEF1* expression, measured by qPCR and considered as a continuous variable, associated with higher CR rate ($P = .05$) and longer RFS ($P = .001$), OS ($P = .005$), and EFS ($P < .001$; supplemental Table 1). To facilitate further validation and potential clinical application of *LEF1* expression as a prognostic marker, we identified an informative threshold (a $\Delta\Delta C_T$ value of -2.5) for stratifying patients according to *LEF1* transcript levels. *LEF1*^{high} expressers, as defined by qPCR, had a trend toward a higher CR rate ($P = .07$) and longer RFS ($P < .001$; Figure 4A), OS ($P = .002$; Figure 4B), and EFS ($P < .001$; Figure 4C) than *LEF1*^{low} expressers (supplemental Table 6). The limited number of patients available for qPCR analysis restricted our ability to study subgroups or evaluate multivariable models. However, in a model for EFS, high *LEF1* expression by qPCR significantly associated with favorable outcomes ($P = .05$) after adjusting for age, sex, WBC, *NPM1*, and *FLT3*-ITD mutational status, *CEBPA* mutations, and *ERG*, *BAALC*, and *MN1* expression (supplemental Table 7).

High *LEF1* expression associates with expression of lymphoid antigens in CN-AML

To gain further insights into biologic differences that are associated with varying *LEF1* expression levels in CN-AML patients, we studied global gene expression profiles. We identified a signature of 4958 genes whose expression significantly correlated with *LEF1* transcript levels, 1885 genes showing a positive correlation and 3073 genes showing a negative correlation (Figure 5, supplemental Table 8). One of the genes most closely correlated with *LEF1* was *TCF7*, encoding the TCF1 protein, another member of the LEF1/TCF-transcription factor family. Notably, other high-ranking genes showing a strong positive correlation with *LEF1* expression encode surface antigens of T lymphocytes, including *CD2*, *CD3*, *CD5*, *CD6*, *CD8*, *CD247* (the T-cell receptor ζ chain), and *IL2RB* (the interleukin-2 receptor β chain). These results indicate that high expression of *LEF1* is associated with up-regulation of genes linked to lymphoid phenotypic differentiation.

Gene set enrichment analysis (GSEA), using a collection of 794 gene sets representing functional biologic pathways, was used to explore biologic mechanisms that might link *LEF1* expression to patient outcomes (supplemental Table 9). Eleven gene sets were significantly associated with high *LEF1* expression, and most of them are associated with T-lymphocyte differentiation and activation. Expression of 41 gene sets was significantly associated with low *LEF1* levels, including gene sets linked to DNA replication, cell-cycle progression, mitosis, and DNA repair. Flow cytometric data from 124 patients confirmed that patients with high *LEF1* had a higher percentage of cells staining positive for CD2 ($P < .001$; Figure 6A) and cytoplasmic CD3 (cyCD3; $P < .001$; Figure 6B). Expression of CD2 in > 20% of cells was observed in 13% of *LEF1*^{high} and 6% of *LEF1*^{low} patients. Expression of cyCD3 in > 10% of cells was observed in 40% of *LEF1*^{high} patients, compared with 30% of *LEF1*^{low} patients.

Discussion

Our study is the first report on the prognostic relevance of *LEF1* expression in AML, and demonstrates that high *LEF1* expression is associated with favorable CR rate, RFS, OS, and EFS in CN-AML. *LEF1* is down-regulated in CD34$^+$ cells from patients with myelodysplastic syndromes (MDS) compared with healthy individuals, and lower *LEF1* expression associates with increasing BM blast counts and disease progression toward AML.[30] In agreement with these results, we found that CN-AML patients with low *LEF1* expression had higher WBC and BM blast percentages and more aggressive disease. In our study, high *LEF1* expression associated with absence of *FLT3*-ITD and low *ERG* expression, which both are favorable molecular characteristics in CN-AML.[19,25,26,31]

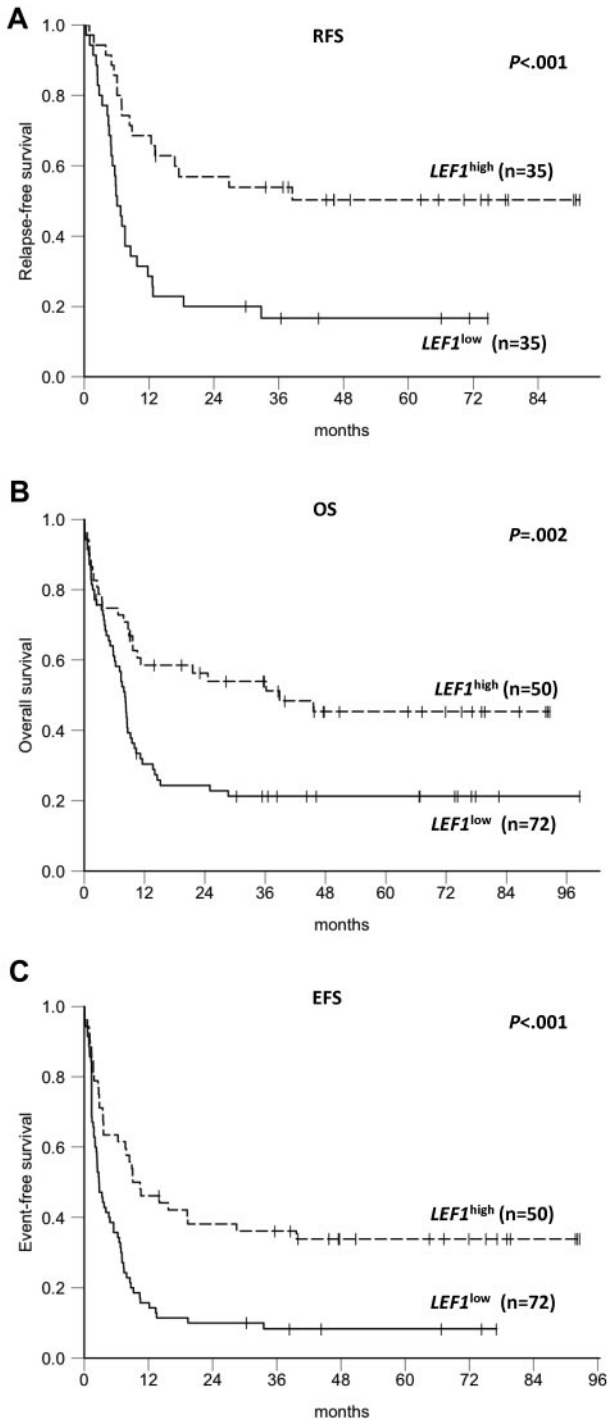

Figure 4. Survival of CN-AML patients according to *LEF1* expression levels measured by quantitative PCR. (A) RFS, (B) OS, and (C) EFS in 122 patients from the primary cohort with available material for qPCR analysis. A $\Delta\Delta C_T$ value of −2.5 was used as the threshold between high and low *LEF1* expressers.

LEF1^{high} CN-AML patients more often belonged to the favorable genetic group as defined by the ELN classification than *LEF1*^{low} patients, but subgroup analyses showed that high *LEF1* expression associated with favorable survival within both genetic subsets. Furthermore, the association of *LEF1*^{high} status with longer RFS, OS, and EFS was confirmed in multivariable analyses adjusting for the most important clinical and molecular prognosticators in CN-AML. These results indicate that low *LEF1* expression is not merely a surrogate marker for other unfavorable genetic lesions such as *FLT3*-ITD.

Our primary cohort included CN-AML patients across a broad age range, and more than one-half of our patients were aged 60 years or older, the age group where most cases of AML occur.[32] Although the prognostic impact of molecular markers can differ between younger and older patients,[28] *LEF1*^{high} status was a favorable prognostic marker in both age groups. High *LEF1* expression also associated with longer OS in a second, independent validation cohort of younger CN-AML patients treated on a different protocol and studied by independent investigators. This finding further supports the validity of our results.

Our initial analyses were based on data from gene expression microarrays, which are difficult to use in a clinical setting. However, we were able to reproduce our results when *LEF1* expression was measured by real-time qPCR, a routine technique in most diagnostic laboratories. We defined a clinically meaningful threshold for *LEF1* expression, which will facilitate further validation of our results by independent investigators and potentially allow future clinical application of this novel genetic marker. Our results suggest that the prognostic impact of *LEF1* expression is most pronounced in the ELN intermediate-I genetic group, and thus *LEF1* expression may be used to further refine risk stratification for these patients. However, further studies in large and molecularly well-characterized cohorts are needed to develop and validate such an improved risk classification system for CN-AML.

The mechanisms underlying the association between high *LEF1* expression and favorable treatment outcomes are unclear. However, our results are of particular interest because *LEF1* is an important downstream effector of Wnt signaling, a pathway that is required for self-renewal of normal hematopoietic and leukemic stem cells.[2,33,34] Leukemogenic fusion genes and gene mutations can induce Wnt signaling in AML, aberrant activation of the Wnt effector β-catenin has been detected in primary AML samples, and small-molecule Wnt pathway inhibitors are cytotoxic for AML blasts.[3,5,35] Our group previously showed that overexpression of *Lef-1* in murine BM leads to disturbed hematopoiesis and, ultimately, to the development of myeloid and lymphoid leukemias.[10] Interestingly, the myeloid leukemias arising in this model originated from a leukemic stem cell with lymphoid characteristics and showed coexpression of lymphoid markers. In our present study, we analyzed genome-wide gene expression profiles to identify biologic pathways that are associated with *LEF1* expression in CN-AML. In line with our observations in mice, *LEF1*^{high} patients also showed up-regulation of gene sets related to T-lymphoid differentiation. On the other hand, gene sets related to cell proliferation, DNA replication, and DNA repair were down-regulated in *LEF1*^{high} patients, which might contribute to their favorable outcomes.

Considering reports that linked Wnt pathway activation and high β-catenin expression with inferior patient outcomes,[2,36] it might seem counterintuitive that increased levels of the β-catenin interaction partner *LEF1* associate with favorable outcomes in CN-AML. There are several possible explanations for our findings: first, the LEF1/TCF-family comprises at least 4 different transcription factors with redundant roles with regard to Wnt signaling.[37] *LEF1* expression is not closely correlated with Wnt pathway activation as assessed by a LEF1/TCF-reporter assay in primary AML blasts.[3] We found that *FLT3*-ITD mutations associated with low expression of *LEF1*, while the Wnt pathway generally is activated in such patients.[38] Moreover, expression levels of *TCF7,* another LEF1/TCF protein, showed a much weaker association with patient outcomes than *LEF1* expression (data not shown). Therefore, patient outcomes in CN-AML may be specifically

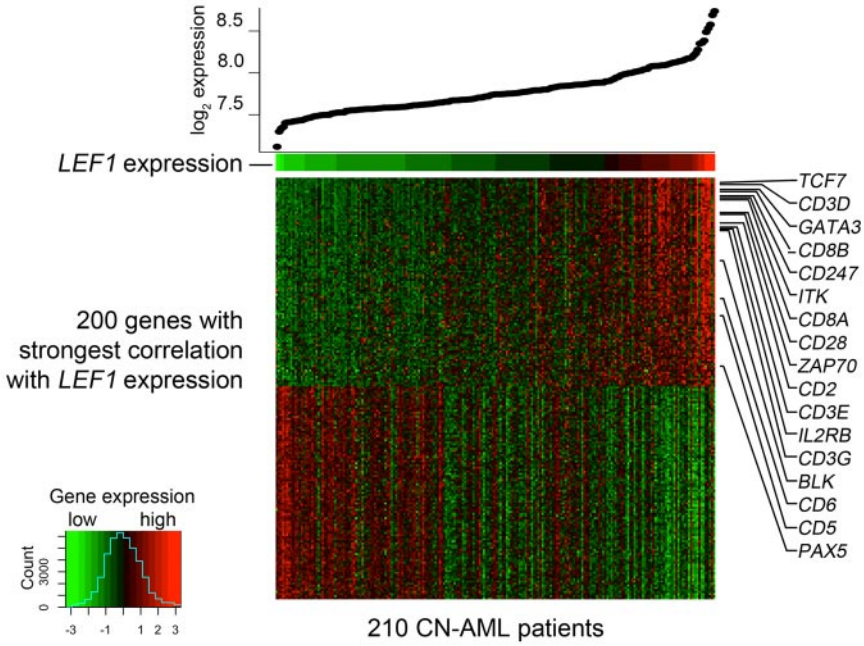

Figure 5. Heatmap image of the 200 genes showing the strongest absolute correlation with *LEF1* expression levels. Each column represents 1 of the 210 CN-AML patients, ordered from left to right according to increasing *LEF1* expression. *LEF1* expression levels are displayed on top of the heatmap. Each row represents 1 gene, ordered from top to bottom according to their Spearman correlation coefficient in descending order.

associated with *LEF1* expression, rather than with deregulation of Wnt pathway activity in general. Second, apart from its involvement in Wnt signaling, *LEF1* is also involved in multiple other cellular pathways.[8,39] *LEF1* is a crucial transcription factor in neutrophilic granulopoiesis. *LEF1* expression is low or absent in patients with severe congenital neutropenia, leading to down-regulation of *CEBPA* and to a block of neutrophilic differentiation.[39] Thus, low *LEF1* expression may also contribute to the differentiation block in MDS and AML blasts, as reflected by the higher WBC and blast percentages in *LEF1*^{low} CN-AML and MDS.[30] Finally, *LEF1* is not only important for granulopoiesis, but is also involved in B- and T-lymphocyte development.[40,41] The causes and effects of abnormal *LEF1* expression likely depend on the cellular context and differentiation stage. The diverse functions of *LEF1* in normal and malignant hematopoiesis are reflected by recent reports that inactivating *LEF1* mutations occur in T-cell acute lymphoblastic leukemia,[42] while high *LEF1* expression is associated with inferior outcomes in B-cell acute lymphoblastic leukemia.[43]

In summary, our study for the first time provides evidence that high *LEF1* expression associates with favorable outcomes in adult CN-AML patients, even after adjusting for known clinical and molecular risk factors. *LEF1* expression can easily be measured by qPCR, and thus may be a valuable new marker for risk stratification of younger and older CN-AML patients. Moreover, our gene expression data from a large cohort of primary CN-AML patients provides insights into the biologic changes associated with varying *LEF1* expression levels in AML, and may trigger further mechanistic studies on the role of *LEF1* in myeloid leukemias.

Acknowledgments

This work was supported by a grant from Deutsche Forschungsgemeinschaft (DFG; SFB 1074, project A4, C.B.) and the Bundesministerium für Bildung und Forschung (NGFN2 grant 01GS0448, C.B.).

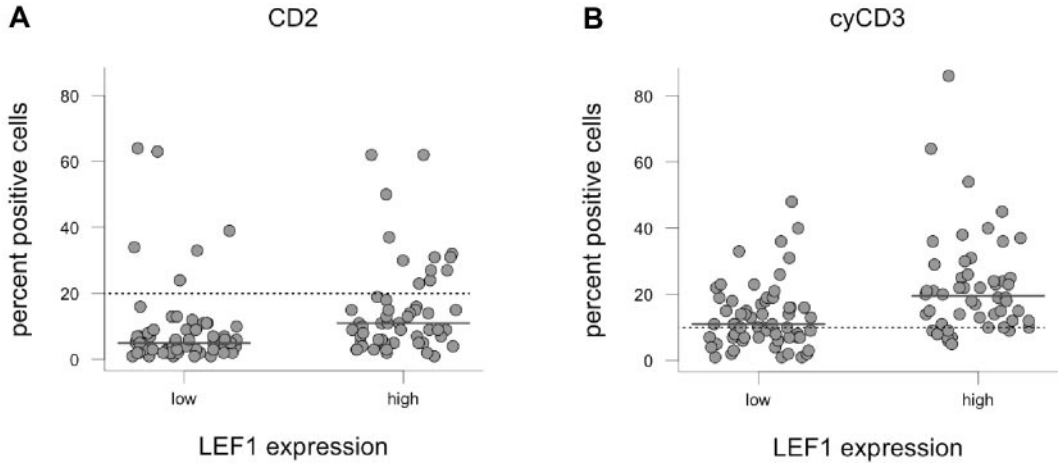

Figure 6. Expression of lymphoid antigens in *LEF1*^{high} CN-AML. (A) CD2 and (B) cytoplasmic CD3 (cyCD3) expression, assessed by flow cytometry, in patients with low and high *LEF1* expression. Each dot represents a patient, and the y-axis shows the percentage of cells within the blast gate staining positive for the respective marker. The red horizontal lines indicate the median for each of group, and the dotted line marks the cutoff for marker expression according to the European Group for Immunophenotypic characterization of Leukemias (EGIL) criteria.

Authorship

Contribution: K.H.M., B.H., L.B., and C.B. designed the study; K.H.M., M.C.S., and R.F.S. performed statistical analyses; K.H.M., B.H., R.F.S., L.B., and C.B. wrote the manuscript; and all authors participated in collecting clinical and/or molecular data, helped analyze and interpret data, and reviewed and approved the final version of the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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